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Powdered Inoculum: A New Approach for Improving Bioethanol Production During Simultaneous Saccharification and Fermentation (SSF) of Lignocellulosic Biomass

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This study presents a practical approach to enhancing microbial inoculum availability through the development of powdered inoculum for use in simultaneous saccharification and fermentation (SSF) of lignocellulosic materials for bioethanol production. The powdered inoculum is composed of a consortium of three microbes: *Trichoderma reesei*, *Saccharomyces cerevisiae*, and *Zymomonas mobilis*. Rice, corn, and soybean flour, supplemented with 5% (w/w) glucose, serve as carriers for the microbial consortium. The stability and viability of the powdered inoculum were evaluated using the Total Plate Count (TPC) method. The inoculum was applied to lignocellulosic material in the form of Dewaka banana pseudostem (DBP), which underwent pretreatment involving chemical-free pressure heating and drying. The powdered inoculum was applied to the lignocellulosic material for a 15-day fermentation period. Parameters monitored included optical density, reducing sugar levels, and bioethanol concentration. The results showed that microbial growth in the inoculum at the time of preparation was 1.26×10^7 CFU g^{-1}, and after four weeks of storage, it was 9.42×10^8 CFU g^{-1}. The bioethanol yield was 34.85 g g^{-1} substrate on the third day of inoculation, with a bioethanol concentration of 12.25% achieved by the 12th day.

Keywords: Powdered inoculum, simultaneous saccharification and fermentation (ssf), bioethanol production, lignocellulosic biomass, microbial inoculum.

INTRODUCTION

Currently, fossil fuels are the most widely used source of energy. However, despite increasing consumption rates in Indonesia and globally, production levels are decreasing. This widening gap between production and consumption highlights the need for alternative energy sources, such as biomass. Bioethanol is a type of fuel produced from biomass. The process of creating bioethanol from biomass involves three distinct stages: pretreatment, hydrolysis, and fermentation. During the pretreatment stage, cellulose is separated from hemicellulose and lignin. The hydrolysis stage follows, where the cellulose is converted into sugar (also known as saccharification), which is then fermented to produce bioethanol. Producing bioethanol from biomass is a lengthy and expensive process. Originally, it involved separate treatments for hydrolysis, fermentation, and pretreatment. However, for greater efficiency and better time management, bioethanol production now utilizes an integrated hydrolysis and fermentation process. Efficient simultaneous saccharification and fermentation (SSF) in high solids is one of the keys to the successful commercialization of lignocellulose-based bioethanol manufacturing processes. Based on techno-economic calculations, the SSF process for bioethanol production from lignocellulosic materials is designed with (1) the fermentation of C_5 and C_6 sugars by a consortium of microbes, and (2) a cellulose and hemicellulase enzyme mixture that works synergistically in the saccharification process (Khajeeram and Unrean, 2017). These processes have been developed by utilizing several types of microbes simultaneously, either in the form of microbial consortia or recombinant microbes (Gusakov,

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2011; Vats *et al.*, 2013; Kricka *et al.*, 2015; Bu *et al.*, 2019). Microbes, whether as microbial consortia or recombinant microbes, are employed by leveraging their differing characteristics. The use of promising microbial consortia faces challenges related to providing microbes that require specific conditions, such as those found in laboratory settings. One approach to address this challenge is to temporarily weaken the microbes in the form of inoculum, similar to the yeast used in the production of tape and tempeh. For generations, inoculum/yeast has been a Tape and tempeh have been fermented with inoculum/yeast for millennia. A consortium of microorganisms, like tape or tempeh yeast, can replace laboratory-prepared inoculum or starter cultures. Previous study has used powdered inoculum to decompose lignocellulosic materials into bioethanol. The carrier material for this powdered inoculum is corn, tapioca, rice, and wheat flour. For bioethanol production from discarded newspaper, *S. cerevisiae*, *Z. mobilis*, and *K. marxianus* are utilized (Safitri *et al.*, 2017). A medium with required nutrients is used to develop the consortium of microorganisms. A powdered inoculum of *T. reesei*, *S. cerevisiae*, and *Z. mobilis* on rice, corn, soybeans, and 5% (w/w) glucose can be used to make bioethanol. The powdered inoculum will be evaluated on chopped Dewaka banana pseudostem (DBP) pretreated with pressure heating for 2 hours and distilled water for 24 hours. No chemicals are utilized in pretreatment. Powdered inoculum is 1.26 × 107 CFU/g effective. This study tests this powdered inoculum for bioethanol production from lignocellulosic raw materials. The inoculum's efficacy will be assessed by optical density, decreasing sugar, and bioethanol levels.

MATERIALS AND METHODS

This research uses trial and error to identify the best solution. This includes numerous trials to adjust variables until the desired outcome is reached. This strategy empirically determines the best approach by observing and evaluating experimental findings. The study lasted from August 2022 to May 2023.

***Preparation of carrier materials*:** Figure 1 shows the powder inoculum production method. The procedure involves several stages, beginning with the preparation of the growth medium. This medium is made from coarsely ground rice, corn, and soybeans, which provide essential nutrients for microbial growth (Aljammas, 2021). The raw materials are carefully selected and ground to the desired consistency to create an optimal environment for the inoculum (Veerabadhran *et al.*, 2021). In Figure 1 the carrier materials rice, corn, and soybeans are first cleaned and soaked separately in clean water for 6 hours. After soaking, they are drained and dried until slightly moist, then coarsely ground and dried at 60°C for 8 hours. The processed materials are stored separately until use. Each step of the process is designed to ensure the quality and effectiveness of the final powdered inoculum (Luangthongkam *et al.*, 2021).

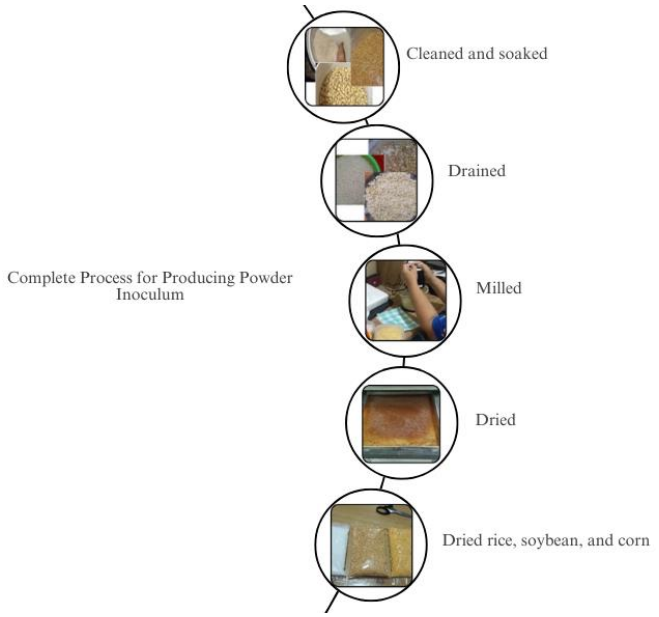

Figure 1. Process for producing carrier materials.

***Microbial Propagation*:** The next stage is microbial propagation (see Figure 2). Pure cultures of *T. reesei*, *S. cerevisiae*, and *Z. mobilis*, obtained from the Food and Nutrition Culture Collection (FNCC) at the Center for Food and Nutrition Studies, Gadjah Mada University, are used. Potato Dextrose Broth (PDB) and Nutrient Broth (NB), both Merck products prepared according to packaging instructions, serve as the growth media. After sterilization, these media are inoculated with the pure cultures at a 1:9 ratios. *S. cerevisiae* is incubated at 30°C for 2 days in PDB, *Z. mobilis* at 37°C for 2 days in NB, and *T. reesei* at 25°C for 5 days in PDB.

Powdered Inoculum Preparations: Subsequently, the complete process for making powder inoculum begins with the preparation of a growth medium from pre-processed rice, corn, and soybeans, mixed in a 1:1:1 ratio and supplemented with 5% (w/w) glucose. This mixture is thoroughly combined in an Erlenmeyer flask, sealed with cotton and aluminum foil, and sterilized using an autoclave at 121°C and 15 psi for 15 minutes before cooling. The medium was then aseptically inoculated with 3% (v/v) of *T. reesei*, *S. cerevisiae*, and *Z. mobilis* starters and incubated at room temperature for 48 hours. Following incubation, the inoculum is dried at 40°C for six hours, ground, and sieved to produce the powder inoculum.

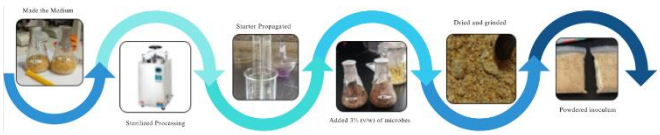

Figure 2. The stages of powdered inoculum manufacture.

***Pretreatment*:** The lignocellulosic material used in this research is Dewaka banana pseudostem (DBP) from Merauke. The pretreatment process for the material used for this test can be seen in Figure 3. First, the DBP is chopped into a uniform size. Next, pressure heating was carried out using an autoclave at a temperature of 121 °C and a pressure of 15 psi for 2 hours. The material remains stored in the autoclave for 12 hours in the cooling process. The material is then removed from the autoclave and soaked in distilled water in a ratio of 1:2 (material and distilled water) for 24 hours. Next, the material was dried at 40 °C for 12 hours and ground.

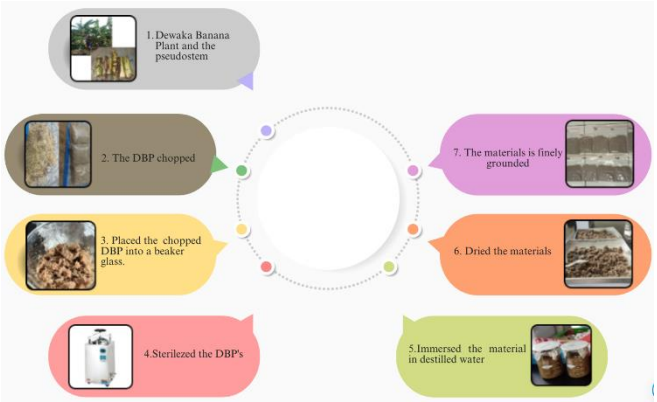

Figure 3. Pretreatment.

***Bioethanol Production Process*:** An overview of the sample inoculation process complete with starter can be seen in Figure 4. The process of making bioethanol begins by preparing the starter. The starter was made by mixing 0.5 gram powdered inoculum, 0.5-gram brown sugar, 3 grams per kilogram of ZA, and 9.0 milliliters of distilled water in a test tube, sealed with cotton wool and aluminum foil. The mixture was homogenized using a vortex. The starter is incubated at room temperature for 2 days and is ready to use. The substrate in the form of dry DBP is placed in a fermentation bottle. Water content was adjusted to 90% by adding distilled water. 2 ml of starter was added for every 30-gram weight of substrate. The process of making bioethanol was carried out for 15 days, with data collection carried out on days 3, 6, 9, 12, and 15.

Each time data was collected, 3 cm^3 of the sample was separated to observe optical density, and another sample was used to observe the levels of reducing sugar and bioethanol. Samples to observe the levels of reducing sugar and bioethanol were centrifuged at 400 rpm for 30 minutes. The supernatant solution was then filtered using Whatman filter paper No. 1. 0.75 ml filtrate is used to measure reducing sugar. Another filtrate will be distilled at 70 °C using a rotary evaporator to determine the ethanol content (Itelima *et al.*, 2013). The optical density value was measured using a SHIMADZU UV-VIS UV-1280 spectrophotometer at a wavelength of 690 nm. Reducing sugar levels were measured using a SHIMADZU UV-VIS UV-1280 spectrophotometer at a wavelength of 510 nm. Bioethanol content was measured using an alcohol meter using dilution theory (Tenkolu *et al.*, 2024).

Figure 4. Inoculation samples with starter.

***Analysis Procedure*:** During the preparation of the inoculum, a test is carried out on the total number of microbes to determine the ability of the microbes to survive in the inoculum. Testing was carried out using the TPC (Total Plate Count) method (Fardiaz, 1993; Safitri *et al.*, 2011). TPC testing was carried out every 8 hours for 2 days. During fermentation, microbial growth is measured using optical density values. The optical density value was measured with a SHIMADZU UV-VIS UV-1280 spectrophotometer at a wavelength of 690 nm. The measured sample is 3 cm3. Blank measurements are carried out by measuring the sample solution that is not inoculated with a starter (Itelima *et al.*, 2013). The testing process for reducing sugar levels begins with the preparation of DNS (dinitrosalicylic acid) reagent and the creation of a standard curve. The sample, consisting of 0.75 ml of filtered supernatant solution and 2.25 ml of DNS reagent, was homogenized. The sample solution was heated to 100 °C for 15 minutes, then allowed to cool to room temperature. A SHIMADZU UV-VIS UV-1280 spectrophotometer with a wavelength of 510 nm was used to measure the absorbance of the sample solution. The absorbance value is used to calculate the concentration of reducing sugar using a previously established standard curve equation. The actual concentration is determined based on the dilution factor (Miller, 1959; Julaeha *et al.*, 2016). Bioethanol levels were measured using an alcohol meter based on dilution theory. The bioethanol to be measured is obtained from distillation using a rotary evaporator at a temperature of 70 °C and a speed of 85 rpm. Calculation of bioethanol content is carried out using the dilution formula. All samples used in this study were repeated twice, and the average values obtained were used for graphical and statistical analysis.

RESULTS

***Microbial Growth in Powdered Inoculum*:** Microbes perform important ecological functions in their life cycle, such as recycling organic materials trapped in cellulose and lignin (Faesal *et al.*, 2020). However, due to its short lifespan, special handling is required to maintain high microbial

viability and effectiveness during storage so that it can be used at any time. Appropriate carrier materials are needed to carry microbes into the biomass to be decomposed. During storage, carrier materials suitable for microbial growth can maintain the high viability and effectiveness of microorganisms. The ideal carrier medium should be non-toxic to microbes, non-abrasive, easy to tamper with and clean, easily accessible, inexpensive, able to store water and contain sufficient nutrients for microbial growth (Djaenuddin *et al.*, 2018). The inoculum that has been made according to the inoculum production procedure in the methodology consists of three carrier ingredients: rice, corn, and soybeans, which are mixed in a ratio of 1:1:1, and the addition of 5% (w/w) glucose. Rice and corn function as sources of carbohydrates, while soybeans function as sources of protein. As is known, the largest component in the elemental composition of microorganisms is carbon. Carbon consists of 50-53% (dry weight) in bacteria, 45-50% (dry weight) in fungi, and 40-63% (dry weight) in molds. Nitrogen is the second most abundant element in microorganisms, constituting 12-15% (dry weight) in bacteria, 7.5-11% (dry weight) in fungi, and 7-10% (dry weight) in molds. Apart from these two elements, microorganisms also contain hydrogen, phosphorus, sulfur, potassium, sodium, calcium, magnesium, chloride, and iron. Other elements (such as Zn, Cu, Mn, Co, Mo, B, and W) are also needed in making growth media (Stanbury *et al.*, 2017a). The elements needed by microorganisms for growth are available in milled rice, yellow corn flour, and soybean flour as listed in the Indonesian Food Composition Table (Direktorat Jenderal Kesehatan Masyarakat, 2018). Carbon, nitrogen, and hydrogen are available in the form of carbohydrates and proteins. In rice, corn, and soybeans, carbon and hydrogen are found in the form of carbohydrates, with amounts of 80, 73.7, and 29.9 mg per 100 gram edible materials (EM), respectively. Nitrogen is available in the form of protein, with rice, corn, and soybeans containing 7.0 mg, 9.2 mg, and 35.9 mg of protein per 100-gram EM, respectively. Other elements such as calcium, phosphorus, potassium, sodium, iron, copper, and zinc are also available in these three materials. The optimal and appropriate inoculum condition is an inoculum with a minimum microbial number of 10^7 CFU gr^{-1}. Therefore, TPC analysis was carried out to assess the growth of the three microbes on the carrier material. Analysis during the inoculum production process was carried out every 8 hours for 2 days, while analysis during storage was carried out every week for a total of 4 weeks. The microbial growth curve on the powder inoculum can be seen in Figure 5. Figure 5 depicts microbial growth on the carrier material. When the three microbes were added to the carrier material, based on the results of the TPC analysis at hour 0, the number of microbes was $2.85x10^7$ CFU gr^{-1}. Furthermore, it was observed that the number of microbes experienced a slight decrease. The decrease occurred from 8 to 24 hours. The decrease in the number of microbes could occur due to the microbial adaptation process to the carrier material.

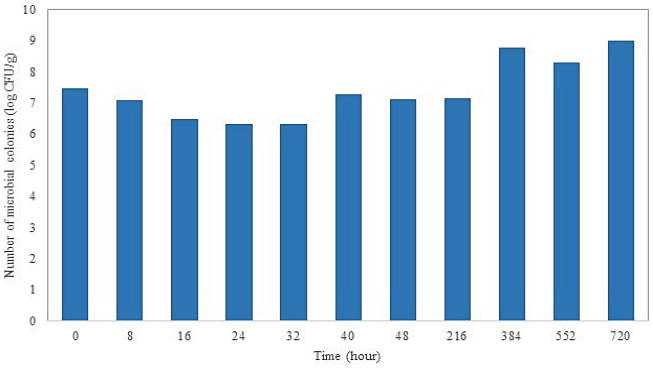

Figure 5. The microbial growth in powdered inoculum.

The number of microbes in the inoculum remained constant after 24 hours and continued to increase thereafter, reaching a microbial number of $1.26x10^7$ CFU gr^{-1}. This number represents the microbial population at the end of powder inoculum production or 48 hours. During storage conditions after 48 hours, an increase in the number of microbes was observed up to $9.42x10^8$ CFU gr^{-1} in the fourth week of storage. This condition can occur due to the general availability of elements needed for microbial growth in the three carrier materials. The main elements required by microbes include carbon, hydrogen, and nitrogen. Likewise, other minerals are needed (Stanbury *et al.*, 2017b; Direktorat Jenderal Kesehatan Masyarakat, 2018). Selection of the most suitable carrier material for the bioethanol production process involves several basic requirements to support the life of the microbes involved in the microbial life process. Microbes need water, an energy source, carbon, nitrogen, mineral elements, vitamins, and oxygen if fermentation occurs aerobically (Stanbury *et al.*, 2017a). The incorporation of microorganisms in carrier materials allows easy handling, long-term storage, and high effectiveness, as observed in the production of biofertilizers (Mukhtar *et al.*, 2017), as well as in the bioethanol production process (Safitri *et al.*, 2017). DBP which has gone through a pretreatment process is used as a medium in this research. Microbial growth in the medium was analyzed by measuring the absorbance of the sample at a wavelength of 690 nm. This measurement is expressed as an optical density (OD) value. Changes in the OD value of the sample can be seen in Figure 6.

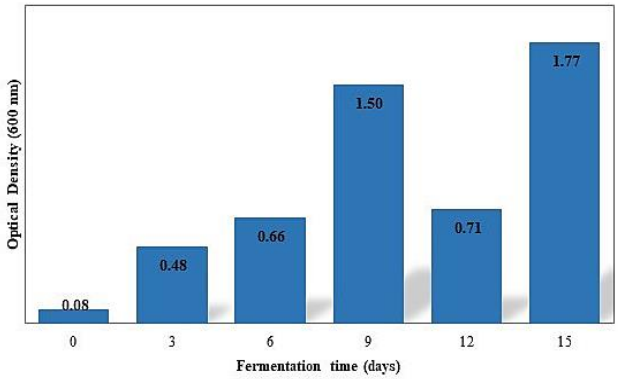

Figure 6. The optical density (600 nm) of the fermentation process.

Figure 6 shows the microbial growth pattern that can be associated with the availability of various types of sugar in the substrate, which causes diauxic occurrence. Diauxic growth occurs when a microbial population uses two carbon sources, resulting in two-phase exponential growth interrupted by a minimal growth lag phase (Chu and Barnes, 2016). Certain substrates can prevent the expression of genes encoding catabolic enzymes and/or transporter proteins. This is known as diauxic or "catabolic repression". However, these mechanisms vary among bacterial strains and require further research (Buendia-Kandia *et al.*, 2018).

***Bioethanol Production*:** Figure 5 presents the results of bioethanol production based on the yield and concentration of bioethanol. The bioethanol yield is calculated as the total amount of bioethanol produced per unit of raw material used. This involves measuring the total volume of bioethanol generated and relating it to the initial amount of substrate. On the other hand, the bioethanol concentration is determined by measuring the percentage of bioethanol in the final product using an alcohol meter, which provides an accurate quantification of ethanol content.

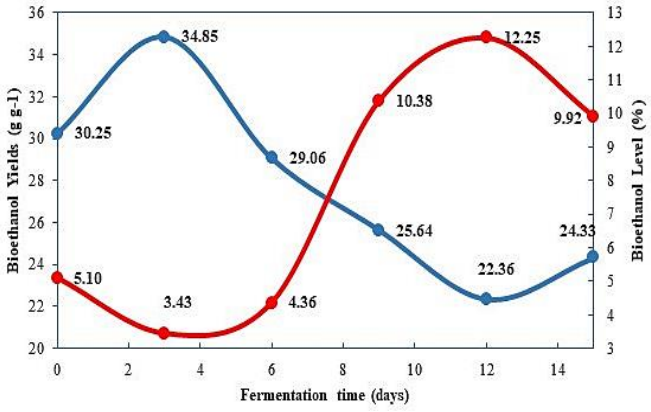

Figure 7. Bioethanol yields (g g^{-1}) and bioethanol level (%).

The graph shows that the third day of fermentation yielded 34.85 g per gram of substrate, indicating an optimum raw material conversion rate. Bioethanol concentration peaked at 12.25% on the 12th day of fermentation. The yield may plateau after an early surge, but the ethanol concentration continues to rise as the fermentation occurs, likely due to the bacteria' continued metabolic activity breaking down the remaining substrates.

DISCUSSION

This study develops a powder inoculum with three bacteria *T. S. reesei. cerevisiae* and *Z. mobilis*. This consortium was chosen because each microbe plays a particular role in hydrolysis and fermentation. *T. reesei* produces cellulase enzymes that hydrolyze cellulose into simple sugars, while *S. Z* and *cerevisiae*. *mobilis* convert sugar into ethanol. In this inoculum, rice, corn, and soybean flour are mixed 1:1:1 and 5% w/w glucose is added. The combination was chosen because it delivers appropriate nutrients and protects bacteria during storage and use. The lignocellulose-based SSF technique tested this powder inoculum. Pressure heating in an autoclave at 121 °C and 15 psi for 2 hours processed lignocellulose without chemicals. After heating, the material is chilled in an autoclave for 12 hours and soaked in 1:2 distilled water for 24. Pretreatment simplifies lignocellulose for cellulase enzymes without producing harmful compounds that impair fermentation (Chukwuma *et al.*, 2020). This chemical-free pretreatment process is simple and eco-friendly (Abolore *et al.*, 2023). Contrary to chemical preparation, chemical residues can be eliminated without washing. It boosts efficiency and sustainability. From lignocellulosic raw materials, this pretreatment procedure yields soluble oligosaccharides, phenolic compounds, and acids for fermentation, similar to LHW. After pretreatment, lignocellulosic material undergoes powder inoculum SSF. T. Reesei produces cellulase enzymes, such as 60-80% cellobiohydrolases, 20-36% endoglucanases, and 1% β-glucosidases. Cellulose is broken down into glucose by enzymes. *Z* and *cerevisiae*. convert glucose to alcohol. Microorganisms functioned for 15 days at 25-30 °C, even though enzymatic hydrolysis was optimal at 50 °C and fermentation was optimal at 30–35 °C.

This study reveals that powdered inoculum can increase microbe life and maintain catalytic activity during storage and fermentation. This idea uses fermented food industry processes like tape or peyeum production to boost bioethanol production efficiency and simplify laboratory preparations. This discovery offers a more practical and sustainable way to produce bioethanol from lignocellulosic biomass. This study found significantly better storage stability with powder inoculum than liquid (Xu *et al.*, 2020). Liquid inoculum needs refrigeration or specific temperatures to preserve bacteria. These parameters are difficult to meet, especially in remote

and huge areas. The powdered inoculum from this study can be stored normally without losing microbial viability. This success relies on microbe dehydration and powdering. Microorganism metabolism and cell component degradation diminish with dehydration (Hamill *et al*., 2020). Bacteria can live longer with lower metabolic activity in poor storage. Mix glucose and rice, corn, and soybean flour 1:1:1 to make powder inoculum. During storage and fermentation, microorganisms can easily use glucose for energy (Sharma *et al*., 2020). This energy source preserves microorganisms throughout storage. Without glucose, microorganisms may lack energy to survive arid conditions (Manzanera, 2021). Carrier grains are rice, corn, and soybean flour.

These ingredients nourish bacteria and support powder inoculum structure. The protein, carbohydrates, and fats in rice, corn, and soybean flour help microorganisms live. Proteins provide amino acids for cell repair and synthesis, carbohydrates provide energy, and lipids preserve cell membranes (de Albuquerque *et al*., 2023). Research shows that nutrient-rich carriers enhance microbial shelf life by providing a stable, nutrient-rich habitat. This study demonstrated that rice, corn, and soybean flour preserved inoculum catalytic activity during fermentation and storage. Example: T's enzymes. Stability keeps reesei, which breaks down cellulose to glucose, high. *S. Z* and *cerevisiae*. mobilis, which ferment glucose to ethanol, remained active. Bioethanol inoculum storage and stability difficulties can be solved with this powdered formulation (Singhania *et al*., 2021). Use of easily available and nutrient-rich carrier materials and glucose as an energy source developed a stable and successful inoculum. Bioethanol production from lignocellulosic biomass is more efficient and sustainable without rigorous storage and laboratory preparation. This investigation found T consortium powder inoculum in SSF. Both *S. reesei. cerevisiae* and *Z. mobilis* produced promising bioethanol from lignocellulosic feedstock. This procedure requires T's enzymes. The composition of reesei contains 60-80% cellobiohydrolases, 20-36% endoglucanases, and 1% β-glucosidases. Ethanol fermentation uses glucose from cellulose decomposition by these three enzymes. T's optimal enzymatic hydrolysis temperature S ferments reesei at 50°C. *Z* and *cerevisiae*. In this study, fermentation lasted 15 days at 25-30°C. Despite low temperatures, the powder inoculum performed well in many conditions. Room-temperature fermentation produces significant reducing sugar, indicating enzyme activity and fermentation at low temperatures (Liszkowska and Berlowska, 2021). Adapting powder inoculum to room temperature is practical, especially in field settings where temperature control is poor (Luangthongkam *et al*., 2021). Stable powdered inoculum lowers storage and transportation concerns and prolongs microbiological vitality. All study suggests powdered inoculum can create bioethanol at scale. Chemical-free pretreatment is sustainable and eco-friendly, making it commercially viable. This study improves bioethanol synthesis from lignocellulosic biomass and encourages additional investigation.

In this study, chemical-free pretreatment revolutionized bioethanol synthesis from lignocellulosic biomass (Rezania *et al*., 2020). Pressure heating like Liquid Hot Water (LHW) changes biomass's chemical structure without injury. This method creates substrates that are easier to process enzymatically without residues, a key benefit. Many benefits come from this chemical-free process. No acids or bases in this procedure reduces pollution and streamlines chemical waste management. Industrial process environmental impact decreases. Second, no washing phase removes residual chemicals, simplifying, speeding, and saving money. Pretreatment without chemicals can alter lignocellulosic biomass's composition (Bhatia *et al*., 2020). Hydrolytic enzymes can reach the substrate after pressurized heating removes lignin and hemicellulose without hurting cellulose. A substrate is easier for the cellulase enzyme to hydrolyze into sugar without producing toxic compounds that inhibit fermentation. This method competes with alkaline pretreatment for biomass-to-sugar conversion. Without chemicals or expensive washing operations, pretreatment without chemicals can produce sugar yields comparable to alkaline methods. A chemical-free pretreatment method could boost bioethanol production efficiency and sustainability. To optimise pretreatment settings, understand reaction mechanisms, and show its industrial economic and environmental sustainability, this technology needs more research. As shown, this study's chemical-free pretreatment method offers various advantages over chemical-based methods (Diyanilla *et al*., 2020). For bioethanol synthesis from lignocellulosic biomass, chemical-free pretreatment like Liquid Hot Water (LHW) is environmentally benign and practical. Chemical-free pretreatment is environmentally benign because it produces no hazardous chemical waste. Chemical-free pretreatment decreases industrial chemical hazards' environmental and health impacts, improving sustainability. No washing is needed to remove chemical residues from biomass, making chemical-free pretreatment more practicable. It streamlines production and accelerates pretreatment to fermentation. This makes bioethanol production cheaper and more efficient. Without chemicals, pretreatment like LHW in this work can change the chemical composition of lignocellulosic biomass without forming harmful molecules that inhibit fermentation (Alawad and Ibrahim, 2024). This ensures bioethanol is devoid of harmful compounds that could harm humans or fermentation microorganisms. Alkaline and chemical-free pretreatment convert biomass into sugar similarly. This shows chemical-free pretreatment may be superior for bioethanol production. This research uses chemical-free pretreatment methods, especially LHW-like ones, to overcome environmental and technological barriers to bioethanol synthesis from lignocellulosic biomass. This technology's environmental

sustainability, adaptability, and process efficiency make it excellent for industrial development. This study advances powder inoculum and chemical-free pretreatment for bioethanol from lignocellulose production, but technological and environmental obstacles remain to commercialize this technique. Process parameters for simultaneous hydrolysis and fermentation are difficult to optimize. Enzyme and microbial activity during SSF is challenging to balance because to the different optimum temperatures. More research is needed to coordinate enzymatic hydrolysis and fermentation at optimal temperatures using process control or enzyme and microbe engineering. Bioethanol manufacturing requires waste management for environmental sustainability. Wastewater recycling and management are serious tasks (Saravanan *et al*., 2021). Effective, eco-friendly waste processing and recycling technology is needed to mitigate environmental impacts and continue bioethanol production. Stable powder inoculum compositions for industrial use require more investigation. Consortia microbial interactions, carrier material selection, and inoculum generating technologies need improvement. Manufacturing uniformity and success require industrial-scale powder inoculum testing and validation. Research and development can make this powder inoculum and chemical-free pretreatment technique a more sustainable and effective approach to create bioethanol from lignocellulose and reduce raw material dependence. fossil fuels and reducing GHG emissions.

***Conclusion*:** This study developed a powder inoculum with *T. reesei*, *S. cerevisiae*, and *Z. mobilis* bacteria, rice, corn, soybean flour, and 5% w/w glucose. The SSF process uses this inoculum to create bioethanol from lignocellulosic biomass. Studies showed this powder inoculum worked well at room temperature and lasted well in storage. Chemical-free pretreatment using pressure heating, like Liquid Hot Water (LHW), breaks down lignocellulose without harmful chemicals, enabling hydrolysis and fermentation. This powdered inoculum's storage and fermentation flexibility is its main asset for field use. Nutrient-rich carrier materials help microorganisms survive, and chemical-free pretreatment improves biomass-to-bioethanol conversion. Commercial SSF process optimization and waste management are required. This study develops feasible, efficient, and sustainable bioethanol production technology to reduce fossil fuel use and mitigate climate change. This powdered inoculum research for bioethanol production has great potential, but it must overcome many barriers to be practical. Room temperature is not ideal for SSF's enzymatic hydrolysis and fermentation operations, which can reduce efficiency. Improve chemical-free production and pretreatment waste management for environmental sustainability. Research should optimize SSF process settings to match both processes' optimal temperature, find more efficient and environmentally friendly pretreatment procedures, and develop more stable and effective inoculum formulations. This research may increase bioethanol production from lignocellulosic biomass, lower its environmental effect, and provide industrial-scale sustainable solutions to assist renewable energy and greenhouse gas reduction.

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***Ethical statement*:** This article does not contain any studies involving humans or animals.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

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